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Expression of Naked Plasmid DNA Injected into the Afferent and Efferent Vessels of Rodent and Dog Livers

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ABSTRACT

A variety of reporter genes within plasmid constructs were injected into the afferent and efferent vessels of the liver in mice, rats, and dogs. Efficient plasmid expression was obtained following delivery via the portal vein, the hepatic vein, and the bile duct. The use of hyperosmotic injection solutions and occlusion of the blood outflow from the liver substantially increased the expression levels. Combining these surgical approaches with improved plasmid vectors enabled uncommonly high levels of foreign gene expression in which over 15 μg of luciferase protein/liver was produced in mice and over 50 μg in rats. Equally high levels of β-galactosidase (β-Gal) expression were obtained, in that over 5% of the hepatocytes had intense blue staining. Expression of luciferase or β-Gal was evenly distributed in hepatocytes throughout the entire liver when either of the three routes were injected. Peri-acinar hepatocytes were preferentially transfected when the portal vein was injected in rats. These levels of foreign gene expression are among the highest levels obtained with nonviral vectors. Repetitive plasmid administration through the bile duct led to successive events of foreign gene expression. The integration of these findings into laboratory and clinical protocols is discussed.

OVERVIEW SUMMARY

Previously, we have shown that the intraportal injection of plasmid DNA in hypertonic solution leads to high levels of hepatocyte transfection in mice. This report found efficient expression following the retrograde injection of plasmid DNA into the hepatic vein and bile duct. For mice, the retrograde injection of naked DNA into the hepatic vein with occlusion of the portal vein or retrograde injection into the bile duct exhibits up to 10% of transfected hepatocytes. The same results were obtained in rats and qualitatively similar preliminary results were obtained in dogs. A protocol involving catheterization of the bile duct enabled repeat gene injections without additional surgery. The high efficiency of expression in larger animals and the use of relatively accessible vessels such as the hepatic vein or bile duct demonstrates the potential clinical utility of these gene transfer protocols.

INTRODUCTION

We have previously shown that plasmid DNA (pDNA) as expressed in the murine liver after injection into the portal vein, the organ's major afferent vessel (Budker *et al.*, 1996). Highly efficient expression required the use of hypertonic solutions containing heparin and restricting outflow from the liver by occlusion of the hepatic vein. We hypothesized that these conditions increased the openings of the siusoidal fenestrae beyond their normal size of ~100 nm to enable pDNA with an hydrodynamic radius of ~100 nm to pass through (Fraser *et al.*, 1980; Fisherman and Patterson, 1996). The efficiency of luciferase expression *in vivo* was similar to that obtained with the transfection of HepG2 cells *in vitro* using cationic lipids as a delivery system. Injection of a human growth hormone (hGH) expression vector resulted in over 50 ng/ml of hGH and injection of β-galactosidase (β-Gal) expression vector resulted in blue staining in ~1% of hepatocytes throughout the mouse liv-

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ers. These levels of expression from naked pDNA were unprecedented.

The present study explores the use of more accessible vessels such as the hepatic vein and the bile duct for delivering the naked pDNA in mice. Efficient gene expression was obtained using these efferent delivery routes. Occlusion of other vessels to restrict outflow of the injection solutions enhanced, but was not critical for, efficient expression. Repetitive injections into the bile duct were also accomplished. Preliminary results are also presented in larger animals—the rat and dog. The incorporation of these findings into laboratory and clinical protocols is discussed.

MATERIALS AND METHODS

Plasmid constructs

The pCILuc plasmid expresses a cytoplasmic luciferase from the human cytomegalovirus (CMV) immediately early (hCMV IE) promoter. It was constructed by inserting the luc+ gene, an *Nhe* I–*Eco* RI luc+ fragment from pSPLuc+ (Promega, Madison, WI), into the pCI expression vector (Promega). pCILux expresses peroxisomal luciferase under control of the hCMV IE promoter. It was constructed by inserting the luciferase gene (*Hind* III–*Bam* HI fragment from pBlueCMVLux) into the *Sma* I site of pCI. pCILacZ was constructed by placing the *Escherichia coli* LacZ gene (*Pst* I–*Apa* I fragment pBS-RSV-LacZ) into the pCI vector (*Sma* I site). The pCMVGH construct was previously described (Andree *et al.*, 1994).

Injection methods

Plasmid delivery into the hepatic vessels was performed in 6-week-old ICR mice, 2.5- to 6.5-month-old, 200- to 300-gram Sprague-Dawley rats, and beagle dogs. Ventral midline incisions were performed to expose the liver and the associated vessels. The mice were anesthetized with intramuscular injections of 1,000 μg of ketamine-HCl (Parke-Davis, Morris Plains, NJ) and methoxyflurane (Pitman-Moore, Mudelein, IL), which was administered by inhalation as needed. The rats were anesthetized with ether and the dogs were anesthetized with halothane by inhalation. The pDNA was injected in solutions containing 2.5 units/ml of heparin (Lypho-Med, Inc., Chicago, IL) (Qian *et al.*, 1991) and either normal saline (0.9% NaCl) or 15% mannitol in normal saline (Sigma Chemical Co., St. Louis,

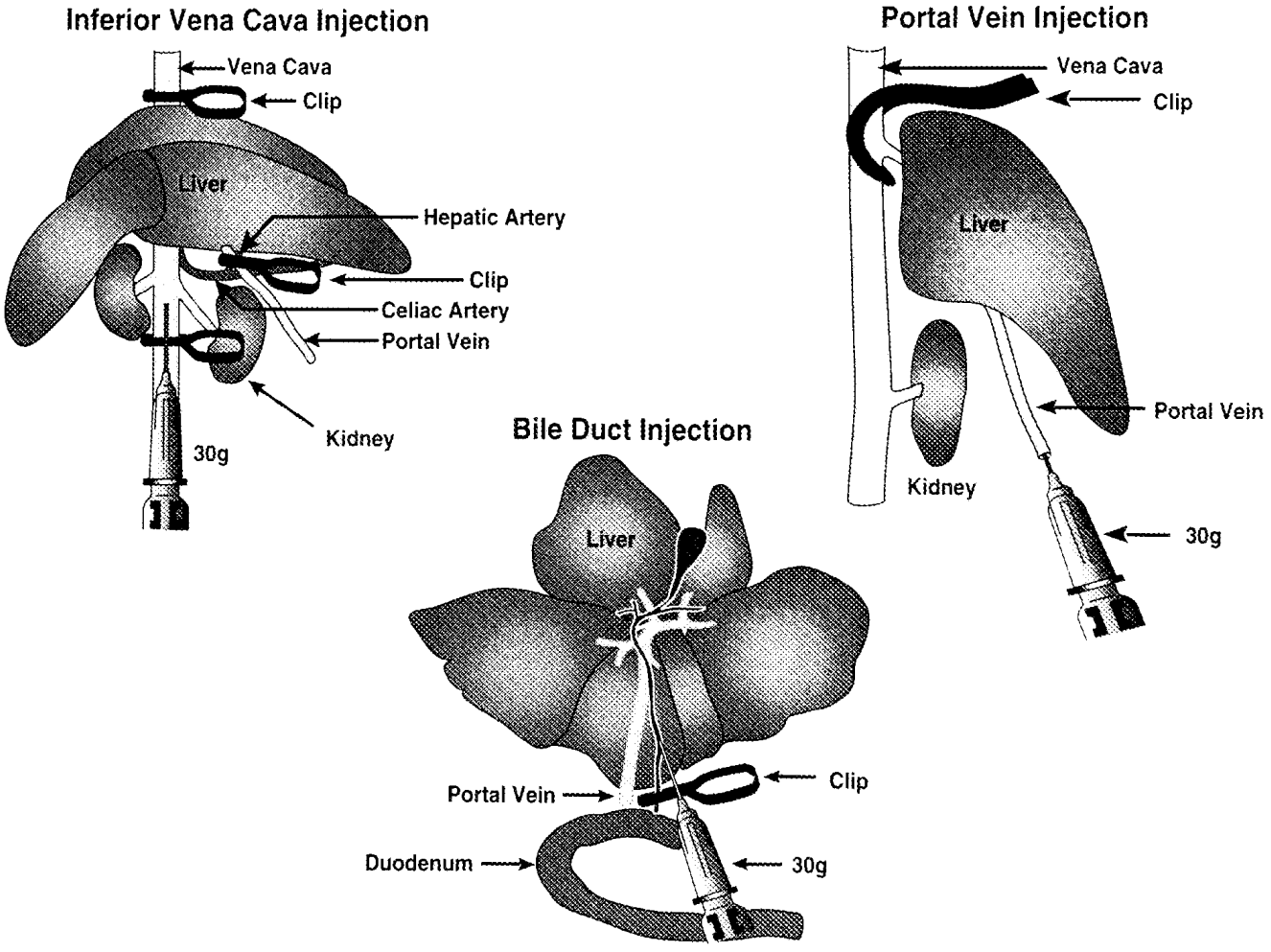

FIG. 1. Comparison of the different injection routes and sites of vascular occlusion.

MO). All animals received humane care in compliance with institutional (IACUC) guidelines.

Figure 1 illustrates the three routes of injection [portal vein, hepatic vein via an occluded inferior vena cava (IVC), and bile duct] and their associated outflow occlusions. In mice, the intraportal injections were performed as previously described (Budker *et al.*, 1996). A total of 100 μg of pDNA in 1 ml was manually injected over ~30 sec using a 30-gauge, 1/2-inch needle and 1-ml syringe without occluding the portal vein upstream from the point of injection. In some animals, a 5 × 1 mm, Kleinert-Kutz microvessel clip (Edward Weck, Inc., Research Triangle Park, NC) was applied during the injection at the junction of the hepatic vein and caudal vena cava.

DNA was delivered in mice to the hepatic vein via an occluded IVC. Clamps (6 × 1-mm, Kleinert-Kutz curved microvessel clip (Edward Weck, Inc., Research Triangle Park, NC)) were applied downstream (toward the heart) of the hepatic vein and upstream (towards the legs) of the hepatic and renal veins. Injections were done upstream of the hepatic vein. In some of the injections, the portal vein and hepatic artery were clamped using 6 × 1 mm, Kleinert-Kutz curved microvessel clips. The IVC mouse injections were also performed with 100 μg of pDNA in 1 ml that was manually injected over ~30 sec with a 30-gauge, 1/2-inch needle and 1-ml syringe.

The bile duct injections in mice were performed using manual injections with a 30-gauge, 1/2-inch needle and 1-ml syringe. A 5 × 1-mm, Kleinert-Kutz microvessel clip was used to occlude the bile duct downstream from the point of injection to prevent flow to the duodenum and away from the liver. The gallbladder inlet was not occluded. In some of the bile duct injections, the junction of the hepatic vein and caudal vena cava was clamped as above. In yet other injections, the portal vein and hepatic artery were clamped in addition to the occlusion of the hepatic vein. Survivability for all three injection routes in mice was almost 100%.

In mice, repetitive injections into the bile duct were done by placing a polyethylene tube (I.D. 0.28 mm, O.D. 0.61 mm; Intramedic Clay Adams Brand, Becton Dickinson Co., Sparks, MD) catheter into the bile duct after making a hole with a 27-gauge needle. The tubing was secured by a suture around the bile duct and tubing, thereby occluding the bile duct. The other end of the tubing was placed outside the skin of the animal's back so that surgery was not required for repeat injections. No blood vessel occlusions were done for these repetitive administrations. After completion of the studies, anatomical examination indicated that the catheter remained in the bile duct.

In rats, the intraportal, IVC, and bile duct injections were done as in mice but with the following modifications. The injections were done through a 25-gauge butterfly needle using a peristaltic pump (Preston varistaltic power pump, Manostat Corp., New York, NY) over 1 or 3 min. The downstream IVC clamps in the IVC injections were done downstream of the kidneys. For the portal vein injections, the portal vein and hepatic artery were clamped. The outflow through the hepatic vein was restricted in some animals by clamping the upstream and downstream IVC. In some animals the livers were first flushed with normal saline prior to DNA injection. Rat bile duct injections were done the same as mice. The rate does not have a gallbladder.

In some of the rat portal vein injections, a 25-gauge needle connected to a pressure gauge (Gilson Medical Electronics, Model ICT-11 Unigraph), was inserted into the liver parenchyma to determine the peak pressure within the liver during the injections. The stastical relationship between pressure and luciferase was done using Spearman's rank correlation. A smoothing spline regression model describing the relationship between log luciferase and pressure was estimated using generalized additive model methodology (Hastie, 1992). Akaike's information criteria (Akaike, 1973) indicated that a smoothing spline with 2 degrees of freedom resulted in the best fit over all integer degrees of freedom between 0 and 5.

The injections in dogs were done as in rats except that an 18-gauge, 2-inch angiocath (Becton Dickinson, San Jose, CA) was used. All dogs except dog #1 were females. Table 1 indicates the injection conditions. For the bile duct injections, a suture was applied to occlude the bile duct transiently downstream from the point of injection. A DeBakey multipurpose vascular clamp was applied to the cystic duct during injection to prevent the injected solution from entering the gallbladder. In dogs, the DNA was pCILux.

Protein assays

The luciferase assays were done as previously reported (Wolff *et al.*, 1990). One day after pCILuc injections, the animals were sacrificed and the rodent livers were divided into six sections composed of right lateral lobe, caudate lobe, two pieces of median lobe, and two pieces of left lateral lobe. For each of the six pieces, 0.7 ml of lysis buffer [0.1% Triton X-100, 0.1 *M* potassium phosphate, 1 m*M* dithiothreitol (DTT) pH 7.8] was used for mice and 4 ml of lysis buffer was used for rat liver. For the dog livers, approximately 10% of each lobe was divided into 5–20 pieces and placed into 2 ml of lysis buffer. The samples were homogenized using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT) and centrifuged at 4,000 rpm for 10 min at 4°C. Twenty microliters of the supernatant was analyzed for luciferase activity. Relative light units (RLU) were converted to picograms of luciferase using standards from Analytic Luminescence Laboratories (ALL, San Diego, CA). The amount of luciferase protein (pg) was calculated using a standard curve in which luciferase protein (pg) = $5.1 \times 10^{-5} \times$ RLU + 3.683 ($r^2 = 0.992$).

Ten-micrometer-thick tissue sections were stained for β-galactosidase (β-Gal) expression as previously described using 1- to 4-hr X-Gal incubations (Budker *et al.*, 1996). Hematoxylin was used for the counterstain but the alkaline step was omitted so that the hematoxylin stain remained red. The percent of blue-stained cells in the liver sections was determined by counting ~3,000 cells in three sections and averaging.

Blood obtained from the retro-orbit sinus was analyzed for serum concentration of hGH using the radioimmune assay (RIA) HGH-TGES 100T kit from Nichols Institute (San Juan Capistrano, CA). Serum ALT and GGT levels were done using EKTACHEM DT slides and a KODAK EKTACHEM DT 60 ANALYZER as recommended by the manufacturer (Kodak, Rochester, NY).

The statistical significance of differences between the means from the two independent samples of growth hormone data was tested with the *t* statistic. The statistical significance of differences for the luciferase data was tested with one-way analysis

of variance (ANOVA) using the SAS generalized linear model procedure because more than two independent samples were involved.

RESULTS

Mice luciferase experiments

Our previous studies used the pBS.CMVLux plasmid (expressing the peroxisomal native luciferase from the CMV promoter) for evaluating the optimal conditions for naked pDNA expressing following intraportal injection. These optimal conditions were intraportal injections of 100 μg of pDNA in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. The injections were done over 30 sec with the hepatic vein and IVC occluded. In this study, 100 μg of pCILuc (expressing a cytoplasmic modified luciferase from a CMV promoter with a chimeric intron) injected under similar conditions yielded a mean total luciferase protein/liver of 3.73 μg/liver (Fig. 2A, PV+CL), approximately 30 times greater than that obtained with pBS.CMVLux. Part of this increase could be attributed to greater operator experience with these injection techniques. Injections with pCILuc under these conditions without clamping the hepatic vein yielded approximately 750-fold less luciferase (Fig. 2A, PV-CL).

The hepatic veins (via the IVC) of another set of mice were injected with 100 μg of pCILuc in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. A mean total luciferase protein/liver of 17.34 μg/liver (Fig. 2A, IVC+CL) was obtained when the portal vein clamped as compared to a mean total luciferase protein/liver of 2.83 μg/liver (Fig. 2A, IVC-CL) without occluding the portal vein.

Similar results were also obtained when bile ducts were injected with 100 μg of pCILuc in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution. A mean total luciferase protein/liver of 15.39 μg/liver (Fig. 2A, BD+CL) was obtained when the hepatic vein was clamped as compared to a mean total luciferase protein/liver of 1.33 μg/liver (Fig. 2A, BD-CL) without occluding the hepatic vein. If mannitol was omitted, then the bile duct injections without clamping any blood vessels yielded approximately 15-fold less luciferase (0.086 μg/liver $\pm$ 0.06, $n = 5$). Clamping the hepatic artery and portal vein in addition to the hepatic vein did not improve expression beyond what was obtained when only the hepatic vein was clamped (data not shown). For all three injection routes, outflow occlusion significantly ($p < 0.05$) increased luciferase expression.

Serum ALT and GGT assays were performed on mice 1 and 8 days after each of the above injections with pCILuc (4 mice for each condition). No increases in GGT were observed after any of the injections, including the bile duct injections. Serum ALT levels increased to 200–400 units/liter 1 day after portal vein and bile duct injections. One day after IVC injections, serum ALT levels increased to ~1,500 units/liter in half of the mice but was only ~250 units/liter in the other half. By 18 days after injection, serum ALT levels decreased to baseline levels in all animals. For positive control purposes, a nonlethal intraperitoneal injection of 40 μl of 50% carbon tetrachloride in mineral oil was performed. An average of 25,900 units/liter ($n = 4$) was observed 1 day after injection. This comparison indicates that the DNA injection procedure caused minimal and transient liver toxicity.

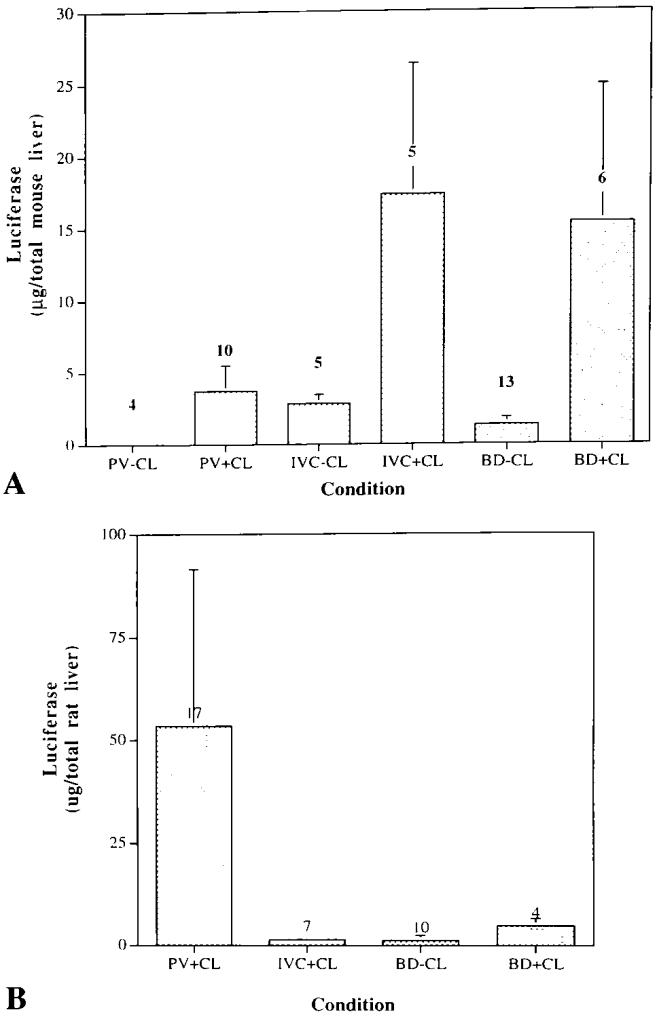

FIG. 2. Comparison of total luciferase expression in mice (A) and rats (B) injected with pCILuc under various conditions. The condition abbreviations signify the following: PV − CL, portal vein injections without clamping; PV + CL, portal vein injections with clamping the hepatic vein; IVC − CL, IVC injections without clamping; IVC + CL, IVC injections with clamping the portal vein and hepatic artery; BD − CL, bile duct injections without clamping; BD + CL, bile duct injections with clamping the hepatic vein. Numbers above the bars indicate the number of mice. T bars indicate the standard error.

Rat and dog luciferase experiments

Similar injections into the portal vein, IVC (to the hepatic vein), and bile duct were done in rats (Fig. 2B). For the portal vein injections, the injection volumes were increased 15 times over that used in mice because the rat livers are ~15 times larger than mouse livers. The amount of pCILuc was only increased ~7.5 times because the use of more pCILuc did not result in significantly more luciferase expression (data not shown). One day after 750 μg of pCILuc in 15 ml of 15% man-

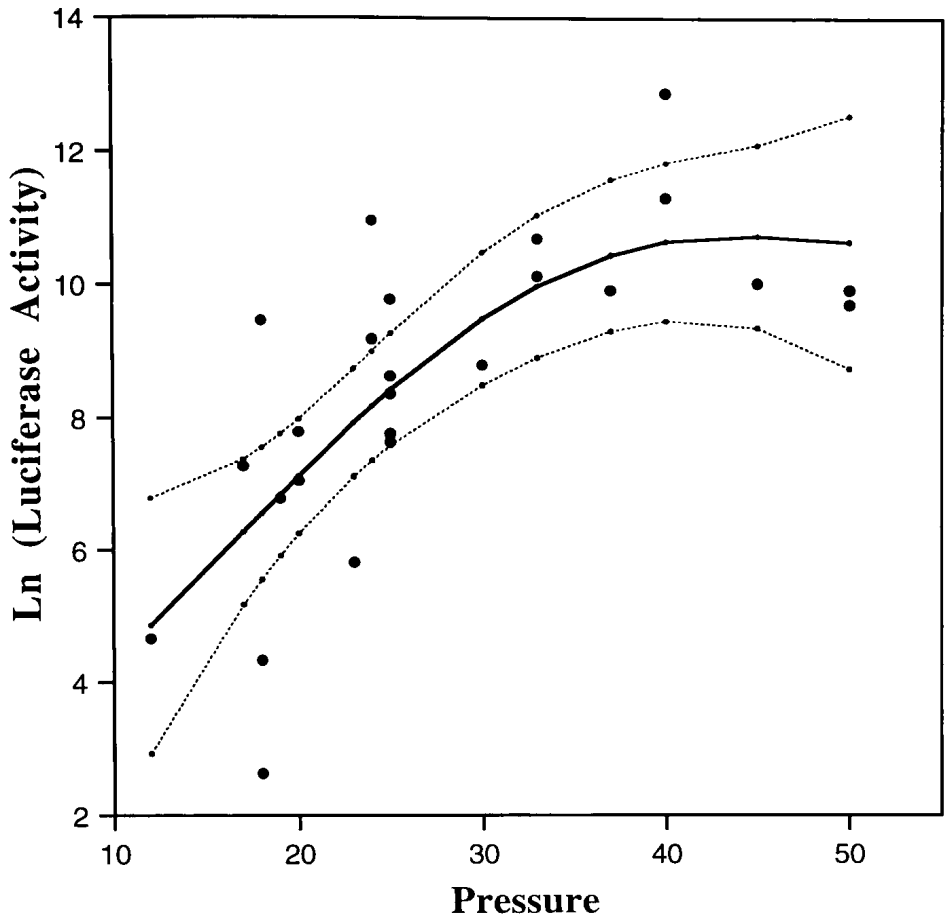

FIG. 3. Scatterplot of the log(e) of luciferase (ng protein) versus peak, intrahepatic, parenchymal pressure (mmHg). The solid line indicates the best fit. Dotted lines indicate 95% pointwise prediction intervals.

nitol and 2.5 units of heparin/ml in normal saline solution were injected into the portal vein while occluding the hepatic vein, an average of 53.5 μg of luciferase/liver was obtained (Fig. 2B, PV+CL). The efficiency of gene transfer in the rat was compared to that in mice in two ways. In terms of efficiency as defined by the nanograms of luciferase per milligram of tissue weight, the levels of expression for portal injections were 3.6 μg of luciferase/gram of tissue in rats as compared to 3.7 μg of luciferase/gram of tissue in mice. Alternatively, in terms of efficiency as defined by the nanograms of luciferase per microgram of pDNA delivered, the efficiencies of expression for portal injections were 71 ng of luciferase/μg of DNA in rats as compared to 37 ng of luciferase/μg of DNA in mice.

Less, but still substantial, luciferase expression was obtained when the injections of pCILuc were done into the efferent vessels of rats such as the IVC or bile duct (Fig. 2B). Injections of 750 μg of pCILuc in 15 ml into the hepatic vein (via the IVC) while occluding the portal vein yielded an average of 1.5 μg of luciferase/liver. Injections of 750 μg of pCILuc in 5–8 ml into the bile duct with outflow obstruction yielded an average of 4.9 μg of luciferase/liver.

Parenchymal pressures of 12–50 mmHg were measured in 23 rat livers during the injection of 750 μg pCILuc in 15 or 20 ml into the portal vein while occluding the IVC (Fig. 3). Spearman's rank correlation between pressure and luciferase expression was 0.76 (two-side p-value <0.001) indicating that pressure and luciferase were significantly positively associated. It appeared that pressures of over 40 mmHg did not result in increased expression. The necessity of the nonlinear component over and above a simple linear fit was verified by an approximate full versus reduced F-test (p value = 0.014), indicating that the observed plateau effect is real. Examination of both the residual quantile-quantile and the residual versus prediction plots further reveals that there are no serious violations of the regression model assumptions and therefore the regression models and p values are valid (Fisher and van Belle, 1993).

Preliminary experiments explored the ability of naked pCILux (not pCILuc) to be expressed in dogs (Table 1). In mice, in the case of intraportal injection, pCILux provided three times lower expression than pCILuc (data not shown). In 5 dogs, various amounts of DNA were injected into either the bile duct with or without blocking outflow by occluding the IVC. In 1 animal, the DNA was injected into the IVC without any outflow blockage. All the dogs survived the procedure except animals that had the IVC occluded recovered more slowly postoperatively. The animals were sacrificed 1 day after the injections and dozens of tissue samples from each liver lobe were analyzed for luciferase. The luciferase expression was evenly distributed over all the lobes in each liver except in one lobe of 1 dog. Routine histological analysis in dog #5 (Table

TABLE 1. LUCIFERASE EXPRESSION FOLLOWING THE INJECTION OF pCILux INTO THE BILE DUCT OR IVC OF DOGS

Dog#	Total LUX (ng)	Liver wt (g)	Dog wt (kg)	DNA amount (mg)	Vessel injected	Out flow block	Inject volume (ml)	Plasmid conc. (μg/ml)	Inject rate (ml/min)
1	182	290.7	8.6	10	Bile duct	no	300	33	75
2	128	357.9	10.8	10	Bile duct	no	300	33	60
3	645	244.2	4.2	14	Bile duct	yes	280	50	70
4	53	430.1	10.4	30	Vena cava	no	600	50	120
5	76	310.2	6.7	20	Bile duct	yes	400	50	100
6	2,961	307.1	6.7	20	Bile duct	no	200	100	66

1) indicated that the tissue architecture was substantially disrupted, suggesting that the injection conditions were not optimal. Decreasing the volume of injection in dog #6 to 200 ml resulted in the best expression (Table 1).

Mice and rat β-Gal results

The β-Gal expression vector was used to determine the percent and type of cells that were transfected (Fig. 4). As previously noted for portal vein injections (Budker *et al.*, 1996), the vast majority of the blue-stained cells appeared to be hepatocytes on morphological grounds but a few appeared to be endothelial or other types of cells. A preponderance of hepatocytes were also stained blue after the bile duct or IVC injections in mice or rats (Fig. 4). No hepatocytes were stained blue after similar injections of pCILuc.

In mice, all three injection procedures resulted in 5–10% of the hepatocytes expressing β-Gal (Fig. 4A–C). In rats, the portal vein injections gave the highest percentage of β-gal-positive cells in which 7% of hepatocytes were positive (Fig. 4D). Much fewer β-Gal-positive cells were noted in rats injected in the IVC (Fig. 4E) or bile duct (data not shown). In some animals, liver cell damage was evident in less than 5% of the cells. Of note in the rat livers injected into the portal vein, almost all of the positively stained cells were peri-acinar with few positive cells around the central vein (Fig. 4D).

Repeat bile duct injections

The bile ducts of mice were cannulated and 100 μg of pCMVhGH in 1 ml of 15% mannitol in normal saline were injected once a week (Fig. 5). Serum levels of hGH increased 1 day after the first injection and then decreased to background levels by 7 days after injection. One day after the second injection, hGH levels again increased and then were back to background levels by 7 days after the second injection. Only minimal increases in hGH levels occurred after the third injection. Mice that had the highest levels after the first injection had the lowest levels after the second injection (mice 3 and 6) and vice versa (mice 1, 2, and 4). Luciferase levels were significantly different between day 1 and day 7 ($p < 0.02$) but were not significantly different between day 9 and day 14 because of the large variability after the second injection. Control mice that received no injections or injections with a luciferase pDNA had mean hGH levels of 0.3 ± 0.1 ng/ml.

In another set of animals (4 mice), the bile duct injections were repeated four times with pCMVhGH and then pCILuc was injected. The first three pCMVhGH injections led to similar increases in hGH serum levels as in Fig. 5. Although there were only minimal raises in hGH serum levels following the fourth injection, injection of pCILuc yielded an average of 29.2 ng/liver (±7.1, $n = 3$). The liver in 1 of the 4 mice was grossly yellow and scarred as a result of the bile duct ligation and did not express any luciferase.

DISCUSSION

This report extends the findings of the previous study showing pDNA expression following afferent intraportal delivery and demonstrates efficient plasmid expression following delivery via efferent vessels such as the hepatic vein or bile duct. Expression of luciferase or β-Gal was evenly distributed throughout the entire liver when either of the three vessels was injected. Combining these surgical approaches with improved plasmid vectors enabled uncommonly high levels of foreign gene expression in which over 15 μg of luciferase protein/liver was produced in mice and over 50 μg in rats (Figs. 2 and 3). Equally high levels of β-Gal expression were obtained in that 5–10% of the hepatocytes had intense blue staining (Fig. 4). These levels of foreign gene expression are among the highest levels obtained with nonviral vectors.

Using the portal vein administration route, occlusion of the outflow is critical for expression. Outflow occlusion increases the expression with the efferent administrative routes, but substantial amounts of expression were obtained even when the hepatic vein was not blocked. Most likely the natural direction of blood flow provides a sufficient impetus to retard the egress of injection fluid and raise the hydrostatic pressure. The use of these efferent vessels simplifies the administration for potential human applications because they are easier to access by noninvasive methods. If no occlusion is used, then only one vessel has to be reached. These efferent routes should also be considered for the administration of viral and nonviral vectors as has been done with the delivery of adenoviral vectors into the bile duct (Yang *et al.*, 1993; Vrancken Peeters *et al.*, 1996a,b).

The mechanism of pDNA uptake is not known but may involve native cellular uptake processes (Budker *et al.*, 1996). It is of interest that high levels of luciferase expression could occasionally be obtained when the DNA was injected into the bile duct in small volumes of isotonic solutions without occluding the IVC. Increased osmolar and hydrostatic pressure may not

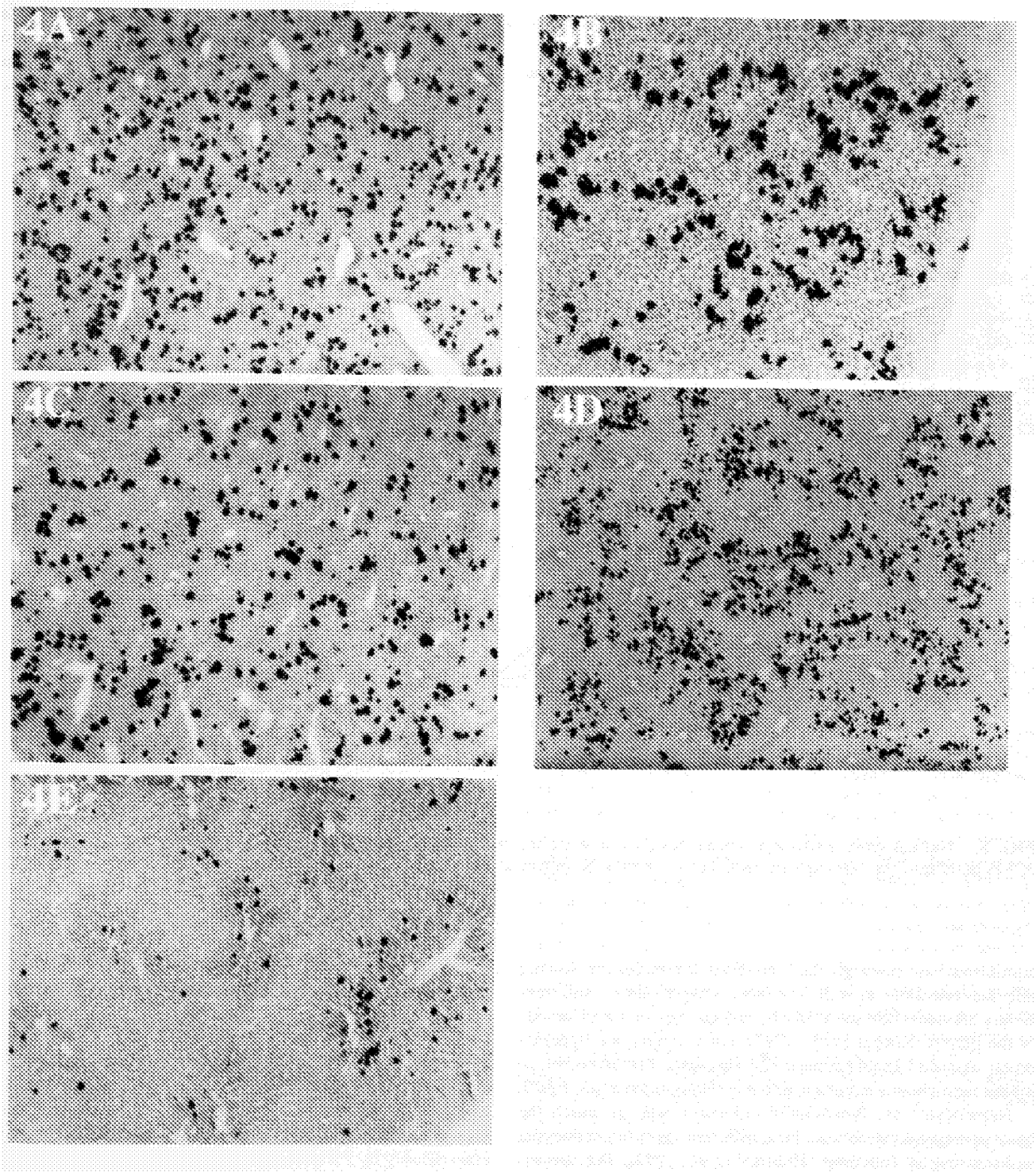

FIG. 4. Histochemical analysis of β-Gal expression in livers after injection of pCILacZ into: mouse portal vein with the hepatic vein clamped (A), mouse IVC with the portal vein and hepatic artery clamped (B), mouse bile duct with the hepatic vein clamped (C), rat portal vein with the IVC clamped (D), and rat IVC with the portal vein and hepatic artery clamped (E). Mouse injections were done using 100 μg of pCILacZ in 1 ml of 15% mannitol and 2.5 units heparin/ml in normal saline solution whereas the rat injections were done using 750 μg of pCILacZ in 15 ml of the same solution. Magnifications, 160× (A and B); 100× (C).

be critical for uptake of the pDNA by hepatocytes, as they are not in muscle cells (Wolff *et al.*, 1990, 1991, 1992a). This would suggest that the mechanism of pDNA uptake may in fact involve endogenous cellular pathways. Increased hydrostatic and osmotic pressures may raise expression by enhancing these cellular internalization processes (Haussinger, 1996).

The raised pressures could also increase the delivery of the pDNA to the hepatocyte surface not only for the blood vessel

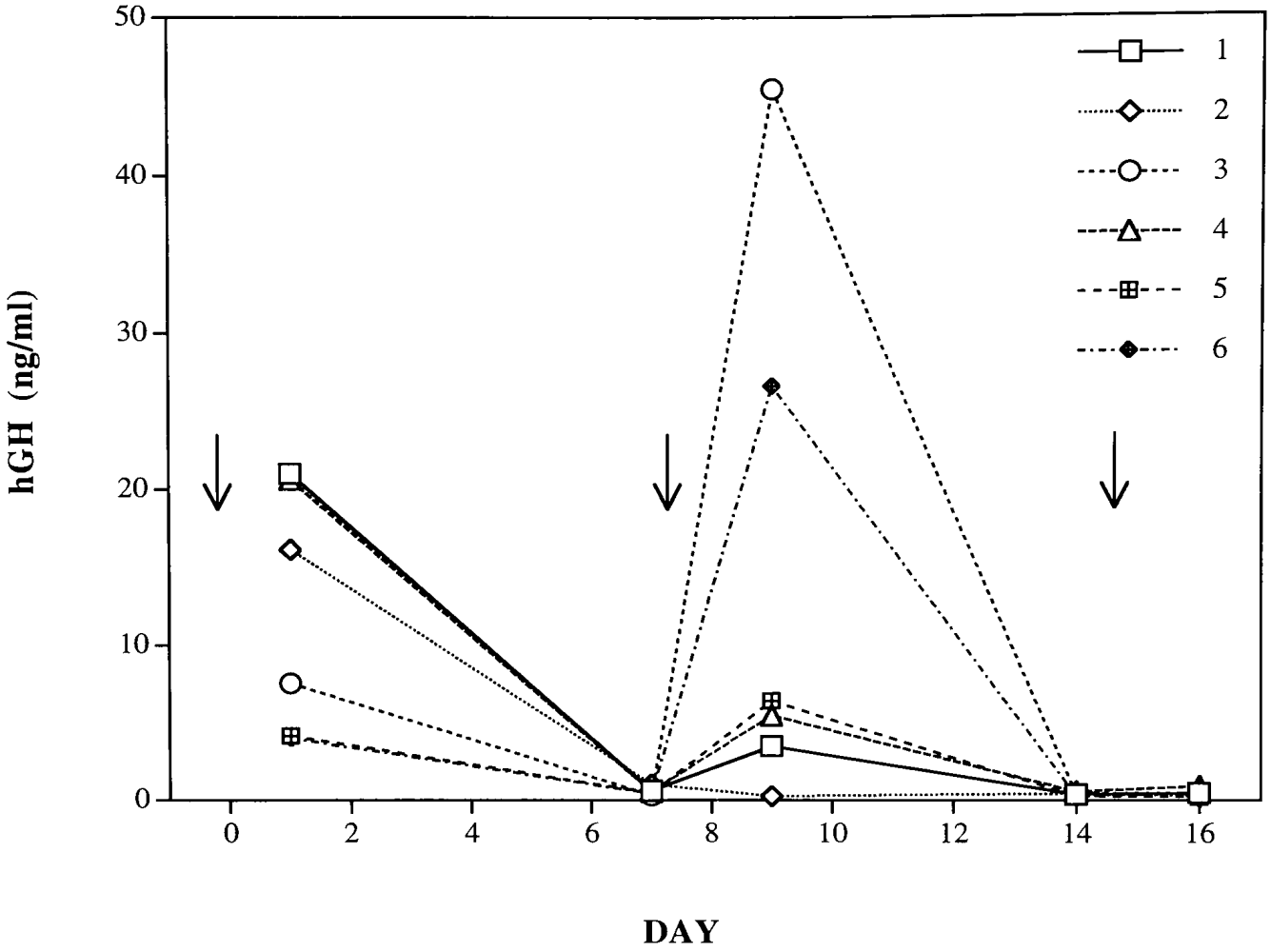

FIG. 5. Human growth hormone (hGH) levels following the repetitive administration (arrows indicates timing) of 100 μg of pCMVhGH into the bile duct of mice via a cannula. Numbers identify individual mice.

administrations (through the sinusoidal fenestrae) but for the bile duct injections as well. The increased pressures could transiently attenuate bile secretion thereby decreasing the clearance of the pDNA (Stieger *et al.*, 1994). For example, the hyperosmolar mannitol should induce cell shrinkage that is known to inhibit taurocholate excretion into bile (Haussinger *et al.*, 1992).

Hepatocytes are functionally polarized cells in which the basal and apical membranes have different exocytic, endocytic, and transcytotic functions (Hubbard *et al.*, 1994). The success with either blood vessel or bile duct routes could indicate that both the basal (sinusoidal) and apical (bile canalicular) membranes share a common pathway for the cellular entry of pDNA. However, the passage of DNA between the basal-lateral and bile canalicular spaces cannot be exluded, even under the more gentle injection conditions. Plasmid DNA injected into the bile duct could be taken up from the basal-lateral surface of hepatocytes after passage paracellularly. If only the basal-lateral surface takes up the pDNA, then the increased hydrostatic and osmotic pressures may enhance pDNA expression by disrupting the tight junctions between hepatocytes and thereby increase the flow of pDNA between the canalicular and basal-lateral spaces (Spray *et al.*, 1994). It is also of interest that the bile duct injections did not result in obvious β-Gal expression in the biliary epithelium as occurs following bile duct injections with recombinant adenoviral vectors (Vrancken Peeters *et al.*, 1996b).

The bile duct was cannulated to determine whether repeat injections could be done. Substantial hGH levels were obtained after the first two injections (Fig. 5). The hGH levels dropped considerably by 1 week after each injection presumably because of an immune response to the foreign protein. The inability to detect hGH after the third or fourth injections was more likely due to a more rapid response of the immune response sensitized to hGH. The ability subsequently to obtain luciferase expression argues against an immune response against the pDNA that prevents expression. Previous studies have failed to detect anti-DNA antibodies following the administration of naked pDNA (Jiao *et al.*, 1992; Nabel *et al.*, 1992). The ability to administer naked pDNA repetitively without inducing an immune response against the vector is a distinct advantage of naked pDNA over viral and some types of nonviral vectors.

Other studies in progress in our laboratory suggest that sup-

pression of the immune system enables more persistent expression. In post-mitotic myofibers, plasmids can persist extrachromosomally and express for at least 2 years, presumably because the pDNA is not being lost as result of cell division (Wolff *et al.*, 1992b; Herweijer *et al.*, 1995). Quite possibly pDNA would be lost slowly in hepatocytes, which have a half-life of up to a year in rodents and humans (Leffert *et al.*, 1988; Webber *et al.*, 1994). If so, the liver-based genetic disorders such as hemophilia could be treated by injections every 6 months. The bile duct could be accessed repeatedly by upper gastrointestinal endoscopy. Similarly, the hepatic vein could be noninvasively accessed via peripheral or central veins. In addition, gene transfer could be delivered to newborns via the umbilical cord vessels to get them over a newborn metabolic crisis, as occurs in the organic acidurias and the urea cycle defects.

Often gene transfer techniques that work in mice do not work in larger animals. Our results demonstrate than the technique works in rats that are approximately 10-fold bigger that mice. The dog results indicate that the liver of larger nonrodent mammal can express naked pDNA. Although substantial levels of luciferase activity were obtained, further optimization of the injection conditions is required to increase the efficiency of expression so that they are comparable to those in rodents. The studies to determine the relationship between intraparenchymal pressure and luciferase expression in rats are a first step toward this goal (Fig. 3). Minimal liver cell damage occurred in the rodents as evident by serum chemistries and histology but the injections were more disruptive to the hepatocytes in dogs. Presumably, the key factor is the efficient delivery of the pDNA to the hepatocyte surface with minimal cellular or tissue disruption.

In the research laboratory, the described techniques will enable rodents to be used just as immortalized and primary liver cell cultures are now used for gene and cellular studies of liver function. The transfer of genes into cells in culture have been a critically important tool for deciphering the function of genes and for studying the effect of expressed proteins on cellular processes. Typically, the gene under study is placed within a plasmid vector and transiently transfected into the appropriate cell in culture. Isoforms and mutant forms of the gene under study can be quickly placed into plasmid expression vectors and studied. Our findings indicate that a similar plasmid-based approach could be used to study the effects of gene function in hepatocytes *in situ*. Given that the high levels of expression are transient in this system, it would be best if these effects occurred within a few days. The use of pDNA avoids the laborious steps necessary for the production of viral vectors or generation of transgenic mice and thereby enables many different genes and their related mutated forms to be quickly studied. It will permit the mechanism of gene expression and their effects on liver function to be expeditiously probed within the context of a complete mammalian organism.

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REFERENCES

AKAIKE, H. (1973). Information theory and an extension of the maximum likelihood principal. In *Second International Symposium on Information Theory*. B.N. Petrov and F. Csaki, eds. (Akademiz Kiado, Budapest) pp. 267–281.

ANDREE, C., SWAIN, W.F., PAGE, C.P., MACKLIN, M.D., SLAMA, J., HATZIS, D., and ERIKSSON, E. (1994). In vivo transfer and expression of a human epidermal growth factor gene accelerates wound repair. Proc. Natl. Acad. Sci. USA **91,** 12188–12192.

BUDKER, V., ZHANG, G., KNECHTLE, S., and WOLFF, J. (1996). Naked DNA delivered intraportally expresses efficiently in hepatocytes. Gene Ther. **3,** 593–598.

FISHER, L.D., and VAN BELLE, G. (1993). *Biostatistics: Methodology for the Health Sciences.* (John Wiley and Sons, New York) chap. 9.

FISHERMAN, D.M., and PATTERSON, G.D. (1996). Light scattering studies of supercoiled and nicked DNA. Biopolymers **38,** 535–552.

FRASER, R., BOWLER, L.M., DAY, W.A., DOBBS, B., JOHNSON, H.D., and LEE, D. (1980). High perfusion pressure damages the sieving ability of sinusoidal endothelium in rat livers. Br. J. Exp. Pathol. **61,** 222–228.

HASTIE, T.J. (1992). Generalized additive models. In *Statistical Models in S.* J.M. Chamber and T.J. Hastie, eds. (Wadsworth & Brooks, Pacific Grove, CA) pp. 247–307.

HAUSSINGER, D. (1996). The role of cellular hydration in the regulation of cell function. Biochem. J. **313,** 697–710.

HAUSSINGER, D., HALLBRUCKER, C., SAHA, N., LANG, F., and GEROK, W. (1992). Cell volume and bile acid extraction. Biochem. J. **288,** 681–689.

HERWEIJER, H., FRITZ, J., HAGSTROM, J., and WOLFF, J. (1995). Direct gene transfer in vivo. In Somatic Gene Therapy P.L. Chang, eds. (CRC Press, Inc., Ann Arbor) pp. 183–202.

HUBBARD, A.L., BARR, V.A., and SCOTT, L.J. (1994). Hepatocyte surface polarity. In *The Liver: Biology and Pathobiology.* 3rd ed. I.M. Arias, J.L. Boyer, N. Fausto, W.B. Jakoby, D. Schachter, and D.A. Shafritz, eds. (Raven Press, New York) pp. 189–213.

JIAO, S., WILLIAMS, P., BERG, R.K., HODGEMAN, B.A., LIU, L., REPETTO, G., and WOLFF, J.A. (1992). Direct gene transfer into nonhuman primate myofibers in vivo. Hum. Gene Ther. **3,** 21–33.

LEFFERT, H.L., KOCH, K.S., LAD, P.J., SHAPIRO, I.P., SKELLY, H., and DE HEMPTINNE, B. (1988). Hepatocyte regeneration, replication, and differentiation. In *The Liver: Biology and Pathobiology.* I.M. Arias, W.B. Jacoby, H. Popper, D. Schachter, and D.A. Shafritz, eds. (Raven Press, New York) pp. 833–850.

NABEL, E., GORDON, D., YANG, Z.-Y., XU, L., SAN, H., PLAUTZ, G., WU, B.-Y., GAO, X, HUANG, L., and NABEL, G. (1992). Gene transfer *in vivo* with DNA-liposome complexes: Lack of autoimmunity and gonadal localization. Hum. Gene Ther. **3,** 649–656.

QIAN, S., FUNG, J.J., DEMETRIS, A.J., ILSTAD, S.T., and STARZL, T.E. (1991). Orthotopic liver transplantation in the mouse. Transplantation **52,** 562–564.

SPRAY, D.C., SAEZ, J.C., Hertzberg, E.L., and DERMIETZEL, R. (1994) Gap junctions in the liver: Composition, function, and regulation. In *The liver: Biology and pathobiology.* 3rd ed. I.M. Arias, J.L. Boyer, N. Fausto, W.B. Jakoby, D.A. Schachter, and D.A. Shafritz, eds. (Raven Press, New York) pp. 951–967.

STIEGER, B., MEIER, P.J., and LANDMANN, L. (1994). Effect of obstructive cholestasis on membrane traffic and domain-specific expression of plasma membrane proteins in rat liver parenchymal cells. Hepatology **20,** 201–212.

VRANCKEN PEETERS, M.-J.T.F.D., LIEBER, A., PERKINS, J., and KAY, M.A. (1996a). Method for multiple portal vein infusions in mice: Quantitation of adenovirus-mediated hepatic gene transfer. BioTechniques **20,** 278–285.

VRANCKEN PEETERS, M.-J.T.F.D., PATIJN, G.A., LIEBER, A., MEUSE, L., and KAY, M.A. (1996b). Adenovirus-mediated hepatic

gene transfer in mice: Comparison of intravascular and biliary administration. Hum. Gene Ther. **7,** 1693–1699.

WEBBER, E.M., WU, J.C., WANG, L., MERLINO, G., and FAUSTO, N. (1994). Overexpression of transforming growth factor-alpha causes liver enlargement and increased hepatocyte proliferation in transgenic mice. Am. J. Pathol. **145,** 398–408.

WOLFF, J.A., MALONE, R.W., WILLIAMS, P., CHONG, W., ACSADI, G., JANI, A., and FELGNER, P.L. (1990). Direct gene transfer into mouse muscle in vivo. Science **247,** 1465–1468.

WOLFF, J.A., WILLIAMS, P., ACSADI, G., JIAO, S., JANI, A., and CHONG, W. (1991). Conditions affecting direct gene transfer into rodent muscle in vivo. BioTechniques **11,** 474–485.

WOLFF, J.A., DOWTY, M.E., JIAO, S., REPETTO, G., BERG, R.K., LUDTKE, J.J., WILLIAMS, P., and SLAUTTERBACK, D.B. (1992a). Expression of naked plasmids by cultured myotubes and entry of plasmids into T tubules and caveolae of mammalian skeletal muscle. J. Cell Sci. **103,** 1249–1259.

WOLFF, J.A., LUDTKE, J.J., ACSADI, G., WILLIAMS, P., and JANI, A. (1992b). Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum. Mol. Genet. **1,** 363–369.

YANG, Y., RAPER, S.E., COHN, J.A., ENGELHARDT, J.F., and WILSON, J.M. (1993). An approach for treating the hepatobiliary disease of cystic fibrosis by somatic gene transfer. Proc. Natl. Acad. Sci. USA **90,** 4601–4605.

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